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Identification of Novel Rodent Herpesviruses, Including the First Gammaherpesvirus of *Mus musculus*▽

Bernhard Ehlers,[1]* Judit Küchler,[1] Nezlisah Yasmum,[1] Güzin Dural,[1] Sebastian Voigt,[2] Jonas Schmidt-Chanasit,[3]† Thomas Jäkel,[4] Franz-Rainer Matuschka,[5] Dania Richter,[5] Sandra Essbauer,[6] David J. Hughes,[7] Candice Summers,[8] Malcolm Bennett,[8] James P. Stewart,[7] and Rainer G. Ulrich[3]‡

P14 Molekulare Genetik und Epidemiologie von Herpesviren, Robert Koch-Institut, Berlin, Germany[1]; FG12 Virale Infektionen, Robert Koch-Institut, Berlin, Germany[2]; Friedrich-Loeffler-Institut, Institute of Epidemiology, Wusterhausen, Germany[3]; German Technical Cooperation (GTZ), GTZ Office Bangkok, Bangkok, Thailand[4]; Institut für Pathologie, Charité Universitätsmedizin Berlin, Berlin, Germany[5]; Institut für Medizinische Mikrobiologie, Infektions- und Seuchenmedizin, Munich, Germany[6]; Division of Medical Microbiology, University of Liverpool, Liverpool, United Kingdom[7]; and Department of Veterinary Pathology, University of Liverpool, Liverpool, United Kingdom[8]

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Rodent herpesviruses such as murine cytomegalovirus (host, *Mus musculus*), rat cytomegalovirus (host, *Rattus norvegicus*), and murine gammaherpesvirus 68 (hosts, *Apodemus* species) are important tools for the experimental study of human herpesvirus diseases. However, alphaherpesviruses, roseoloviruses, and lymphocryptoviruses, as well as rhadinoviruses, that naturally infect *Mus musculus* (house mouse) and other Old World mice are unknown. To identify hitherto-unknown rodent-associated herpesviruses, we captured *M. musculus*, *R. norvegicus*, and 14 other rodent species in several locations in Germany, the United Kingdom, and Thailand. Samples of trigeminal ganglia, dorsal root ganglia, brains, spleens, and other organs, as well as blood, were analyzed with a degenerate panherpesvirus PCR targeting the DNA polymerase (DPOL) gene. Herpesvirus-positive samples were subjected to a second degenerate PCR targeting the glycoprotein B (gB) gene. The sequences located between the partial DPOL and gB sequences were amplified by long-distance PCR and sequenced, resulting in a contiguous sequence of approximately 3.5 kbp. By DPOL PCR, we detected 17 novel betaherpesviruses and 21 novel gammaherpesviruses but no alphaherpesvirus. Of these 38 novel herpesviruses, 14 were successfully analyzed by the complete bigenic approach. Most importantly, the first gammaherpesvirus of *Mus musculus* was discovered (*Mus musculus* rhadinovirus 1 [MmusRHV1]). This virus is a member of a novel group of rodent gammaherpesviruses, which is clearly distinct from murine herpesvirus 68-like rodent gammaherpesviruses. Multigenic phylogenetic analysis, using an 8-kbp locus, revealed that MmusRHV1 diverged from the other gammaherpesviruses soon after the evolutionary separation of Epstein-Barr virus-like lymphocryptoviruses from human herpesvirus 8-like rhadinoviruses and alcelaphine herpesvirus 1-like macaviruses.

Herpesviruses of small laboratory rodents, such as mice and rats, are used as surrogate models of human herpesvirus infections. They are invaluable tools for exploring various aspects of virus-host interactions, which otherwise would be difficult or even impossible to study. Human herpesviruses either do not replicate in laboratory animals or, if they do, frequently fail to cause symptoms that reflect those observed in infected humans. For example, the human varicella-zoster virus (VZV) (species, *Human herpesvirus 3* [HHV-3]; subfamily, *Alphaherpesvirinae*) does not replicate in rodents. Experimental infection of guinea pigs is possible, but its significance is limited (14). Mice, guinea pigs, and rabbits can be infected with herpes simplex virus type 1 (HSV-1) (species, HHV-1; subfamily, *Alphaherpesvirinae*) but do not develop all of the facets of human pathology (13, 28). Experimental infection of New World monkeys with Epstein-Barr virus (EBV) (species, HHV-4; subfamily, *Gammaherpesvirinae*) (10, 20) has been reported, but these animals are endangered, rare, and expensive and as such are of limited experimental value. Infection of laboratory rodents with EBV has not been reported. Therefore, animal homologues of human herpesviruses are required for in vivo studies, and some rodent herpesviruses are currently being studied in detail.

Murine cytomegalovirus (MCMV) (species, *Murid herpesvirus 1* [MuHV-1]; subfamily, *Betaherpesvirinae*) (Table 1) naturally infects *M. musculus* (5). It serves as a tool for experimental studies of human CMV (HCMV) (species, HHV-5; subfamily, *Betaherpesvirinae*) disease (25). For the same purpose, two strains of rat CMV (RCMV) (species, MuHV-2; subfamily, *Betaherpesvirinae*), RCMV strain England (RCMV-E) and RCMV strain Maastricht (RCMV-M), have been investigated in laboratory strains of *Rattus norvegicus* (6, 16). The viruses differ considerably in gene content and biological properties (27, 2).

Murine gammaherpesvirus 68 (MHV-68) (species, MuHV-4;

* Corresponding author. Mailing address: Robert Koch-Institut, Nordufer 20, D-13353 Berlin, Germany. Phone: 4918887542347. Fax: 4918887542598. E-mail: ehlersb@rki.de.

† Present address: Institut für Medizinische Virologie, Klinikum der Johann Wolfgang Goethe Universität, Frankfurt/Main, Germany.

‡ Present address: Friedrich-Loeffler-Institut, Institute for Novel and Emerging Infectious Diseases, Greifswald-Insel Riems, Germany.

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TABLE 1. Known herpesviruses of humans, rodents, and other mammals

Herpesvirus subfamily	Herpesvirus genus[a]	Herpesvirus species in[b]:			
		Humans	*M. musculus*	Rodents	Other mammals
Alphaherpesvirinae	*Simplexvirus*	HHV-1 (HSV-1), HHV-2 (HSV-2)	?	?	Diverse HV
	Varicellovirus	HHV-3 (VZV)	?	?	Diverse HV
Betaherpesvirinae	*Cytomegalovirus*	HHV-5 (HCMV)	?	?	Primate HV
	Muromegalovirus		MuHV-1 (MCMV)	MuHV-2 (RCMV)	
	Roseolovirus	HHV-6, HHV-7	?	?	Primate HV
	Unassigned			CaHV-2 (guinea pig CMV)	TuHV-1
Gammaherpesvirinae	*Lymphocryptovirus*	HHV-4 (EBV)	?	?	Primate HV
	Rhadinovirus	HHV-8 (KSHV)	?	MuHV-4 (MHV-68)[c]	Diverse HV
	*Percavirus**		?	?	Perissodactyl HV, carnivore HV
	*Macavirus**		?	?	Artiodactyl HV
Unassigned			MuHV-3 (mouse thymic HV)	MuHV-5 (*Microtus pennsylvanicus* HV), MuHV-6 (sand rat inclusion agent), CaHV-1 (guinea pig HV), CaHV-3 (Guinea pig HV), CrHV-1 (hamster HV)	Diverse HV

[a] *, proposed genus.
[b] ?, no herpesviruses of *Mus musculus* or other rodent species known at the beginning of this study; HV, herpesviruses.
[c] Also closely related strains, such as MHV-72.

subfamily, *Gammaherpesvirinae*) is a representative of the rhadinoviruses, such as the human Kaposi's sarcoma-associated herpesvirus (KSHV) (species, HHV-8; subfamily, *Gammaherpesvirinae*). MHV-68 is well suited for the study of gammaherpesvirus pathogenesis and was used to develop therapeutic strategies against gammaherpesviruses (reviewed in references 15, 21, and 23). However, MHV-68 pathology in mice does not entirely resemble the pathology of HHV-8 or EBV in humans. For example, MHV-68 infection does not consistently result in tumor development (reviewed in reference 17). In addition, *Mus musculus* (from which laboratory mice are derived) is apparently not the natural host of the virus. MHV-68 and several closely related viruses, such as MHV-60, MHV-72, and MHV-76, were isolated in Slovakia from two rodent species, *Myodes glareolus* (formerly *Clethrionomys glareolus*) and *Apodemus flavicollis* (4). In the United Kingdom, *Apodemus sylvaticus* was found to be the major natural host of MHV-68 (3).

Alphaherpesviruses, roseoloviruses, and lymphocryptoviruses that naturally infect any species of the *Rodentia* are currently not known. In addition, no rhadinoviruses that naturally infect *M. musculus* or other Old World mice have been identified (Table 1).

To identify hitherto-unknown rodent-associated herpesviruses, we captured *M. musculus*, *R. norvegicus*, and 14 other rodent species at several locations in Germany, the United Kingdom, and Thailand and searched by degenerate PCR methods for herpesviruses that naturally infect them. Thirty-eight novel rodent herpesviruses were detected, among them the first gammaherpesvirus of *M. musculus*.

MATERIALS AND METHODS

Sample collection and DNA preparation. Free-living rodents were trapped in several rural and urban locations in Germany, the United Kingdom, and Thailand. A total of 1,132 samples from blood, brain, trigeminal ganglion, spinal ganglion, spleen, lung, intestine, liver, and inguinal lymph nodes were collected and stored at −20°C. DNA was prepared as described previously (26).

Panherpesvirus PCR with specificity for the DPOL gene of herpesviruses. Panherpesvirus consensus PCR for amplification of 160 to 181 bp (without primer-binding sites) of the DNA polymerase (DPOL) gene (Fig. 1A) (8) was carried out as described previously (7). Briefly, PCR was performed with degenerate and deoxyinosine (deg/dI)-containing primers in a nested format. Three primers were used in first-round PCR (primer 285s DFA, 5′-gayttygc[n/I]agyyt[n/I]taycc-3′; primer 285s ILK, 5′-tcctggacaagcagcar[n/I]ysgc[n/I]mt[n/I]aa-3′; primer 285as KG1, 5′-gtcttgctcaccag[n/I]tc[n/I]ac[n/I]ccytt-3′), and two primers were used in second-round PCR (primer 286s TGV, 5′-tgtaactcggtgtaygg[n/I]ttyac[n/I]gg[n/I]gt-3′; primer 286as IYG, 5′-cacagagtccgtrtc[n/I]ccrta[n/I]at-3′). Buffy coat DNA or tissue DNA (250 ng) or products of the first-round PCRs (1 μl) were used as templates in PCRs with a 25-μl reaction mixture containing a 1 μM concentration of each PCR primer (Metabion, Martinsried, Germany), a 200 μM concentration of each deoxynucleoside triphosphate, 1 unit of DNA polymerase AmpliTaq Gold, and 2.5 μl of GeneAmp 10× PCR buffer with 2 mM $MgCl_2$ (Applied Biosystems GmbH, Darmstadt, Germany) and 5% dimethyl sulfoxide (Sigma-Aldrich Chemie GmbH). In first- and second-round PCRs, the reaction mixtures were kept for 12 min at 95°C for activation of the polymerase and then cycled 45 times with 20 s of denaturation at 95°C, 30 s of annealing at 46°C, and 30 s of strand extension at 72°C, followed by a final extension step at 72°C for 10 min.

Samples with little or no amplification product were reanalyzed under more-relaxed conditions; i.e., the ramp time between the annealing step and the extension step was prolonged 50-fold, and the final concentration of AmpliTaq Gold was doubled (2 units/reaction mix). In each assay, a DNA of a gammaherpesvirus-positive porcine spleen sample was included as a positive control. Water samples were extracted and PCR tested as negative controls. In addition, alpha-, beta-, and gammaherpesvirus DNAs were tested at intervals to control assay performance.

Amplification of gB- and MDBP-encoding sequences with degenerate primers. All primers are listed in Table 2. For amplification of novel glycoprotein B (gB) gene sequences of members of the *Betaherpesvirinae* subfamily, three different deg/dI nested-primer sets were used: CM-gB1, CM-gB2, or CM-gB3. Second-round amplification products had sizes of approximately 225 bp, 265 bp, and 280 bp, respectively (without primer-binding sites [Fig. 1A]). For amplification of gB sequences of members of the *Gammaherpesvirinae* subfamily, the deg/dI nested-primer set RH-gB was used. Second-round amplification products had a size of approximately 450 bp (without primer-binding sites [Fig. 1A]). For amplification of sequences encoding the major DNA-binding protein (MDBP) of *Mus musculus*

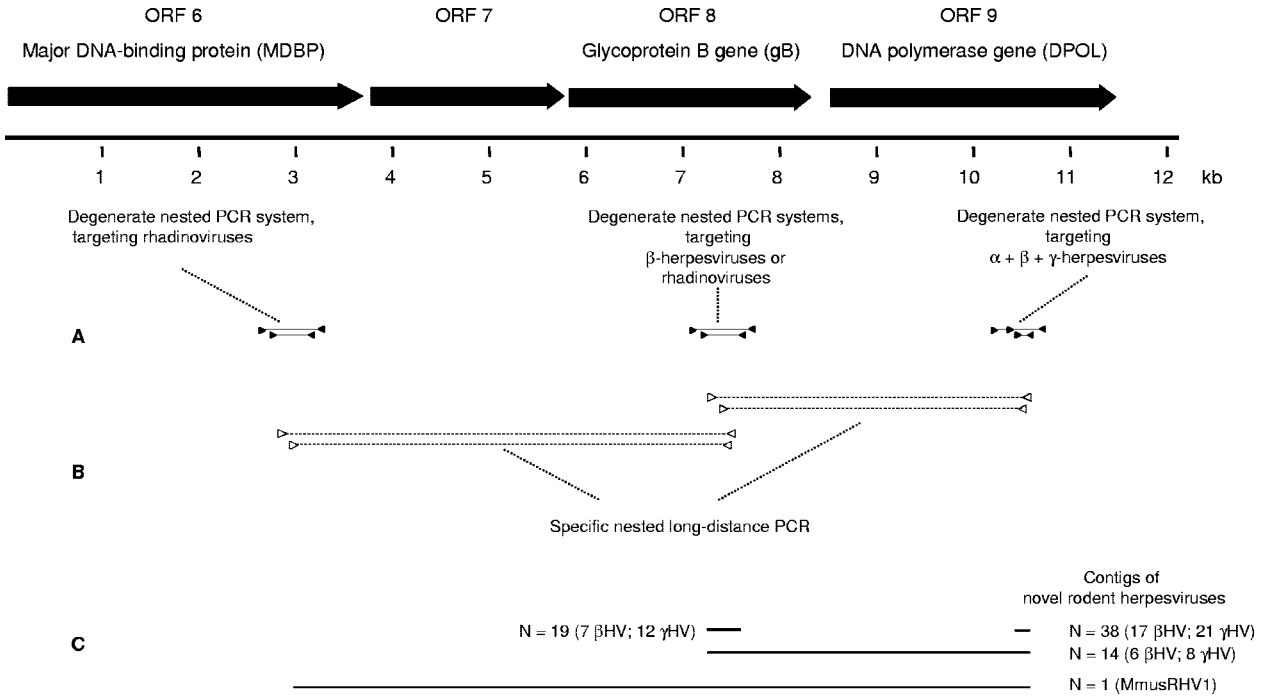

FIG. 1. Map of amplified genes and diagrams of PCR strategies. Degenerate nested primers (black triangles) were used to amplify part of the MDBP gene, the gB gene, or the DPOL gene. (A) The amplified fragments are represented by thin solid lines between the primer-binding sites. (B) Long-distance nested PCR was performed with specific primers (open triangles). The amplified fragments are represented by dashed lines between the primer-binding sites. (C) The number of novel rodent herpesvirus contiguous sequences is specified. Their locations are depicted with thick solid lines. βHV, betaherpesviruses; γHV, gammaherpesviruses. At the top of the figure, the genomic locus spanning ORF6 (MDBP) to ORF9 (DPOL) is depicted with black arrows, indicating the direction of transcription. ORF numbering (ORF6 to ORF9) is adapted from the ORF nomenclature of herpesvirus saimiri (accession no. X64346). The start of the ruler corresponds with the first base of ORF6.

rhadinovirus 1 (MmusRHV1), the deg/dI nested-primer set RH-MDBP was used. Second-round amplification products had a size of approximately 260 bp (without primer-binding sites [Fig. 1A]). PCR was carried out at a 45°C annealing temperature. All other parameters were as described for DPOL gene amplification.

LD-PCR with specific primers. Long-distance PCR (LD-PCR) (Fig. 1B) was performed with the TaKaRa-Ex PCR system (Takara Bio Inc., Japan), according to the manufacturer's instructions. Amplimers were obtained by nested PCR using specific primers (not listed). For the second round, a 1-μl aliquot of the

TABLE 2. Primers used for amplification of novel gB and MDBP sequences

Primer set	PCR round	Primer	Sequence 5′→3′[a]
CM-gB1	1	2743s	CGCAAATCGCAGA(n/i[a])KC(n/i)TGGTG
		2746as	TGGTTGCCCAACAG(n/i)ATyTCrTT
	2	2744s	TTCAAGGAACTCAGyAArAT(n/i)AAyCC
		2745as	CGTTGTCCTC(n/i)CC(n/i)AryTG(n/i)CC
CM-gB2	1	2890s	CTACGACGTGCTCAAGGA(n/i)TAyAT(n/i)AA
		2894as	TGGTGAAACC(n/i)CCrAA(n/i)GGrTT
	2	2891s	CGAAACATCATGGA(n/i)KC(n/i)TGGTG
		2893as	CGTTGTTCTC(n/i)CC(n/i)AryTG
CM-gB3	1	3135s	GTATGATGTGCTGAGGGA(n/i)TAyAT(n/i)AA
		3138as	TCATGAGAAGACC(n/i)CCrAA(n/i)GGrTT
	2	3136s	TTGAGAAACATTTT(n/i)GA(n/i)GC(n/i)TGGTG
		3137as	TCTAAACGTCCCAAGAAG(n/i)ATyTCrTT
RH-gB	1	2759s	CCTCCCAGGTTCArTwyGCMTAyGA
		2762as	CCGTTGAGGTTCTGAGTGTArTArTTrTAyTC
	2	2760s	AAGATCAACCCCAC(n/i)AG(n/i)GT(n/i)ATG
		2761as	GTGTAGTAGTTGTACTCCCTrAACAT(n/i)GTyTC
RH-MDBP	1	3156s	TTTAGGGCCTG yGG(n/i)CarAC
		3159as	GAGTTGCCCGTGAC(n/i)CC (n/i)GTrTA
	2	3157s	CCTGCGGCCAGAC(n/i)CArTTyTA
		3158as	GCCCGTGACCCC AGTrTAyTTrTT

[a] i, inosine.

first-round reaction mix was used as a template. In case of accumulation of nonspecific products in a high-molecular-weight range (>10 kb), the first-round postcycling reaction mix was diluted 1:100 before serving as a template in the second round. Amplification products had a size of approximately 3.5 kbp.

Specific PCR assays. The gB gene of MmusRHV1 was specifically amplified in a nested PCR with primers MmusRHVgB1 (5′-TCGGGAGTATAACTATTACAC-3′) and MmusRHVgB2 (5′-ACCTCCCGAGACTTACTC-3′) in the first round and MmusRHVgB3 (5′-GCCATCATGGAAGACCTG-3′) and MmusRHVgB4 (5′-GAAGAGGATGACGATCAC-3′) in the second round, at annealing temperatures of 53°C and 50°C, respectively. Amplification products had sizes of 317 bp and 131 bp, respectively (without primer-binding sites).

The DPOL gene of *Apodemus flavicollis* cytomegalovirus 3 (AflaCMV3) was specifically amplified with primers 3838s (5′-CAAAGGAAGGCGATTAGACA-3′) and 3838as (5′-ACCGTAACACGCAGTGGAT-3′) at a 57°C annealing temperature. Amplification products had a size of 254 bp (without primer-binding sites).

Cytochrome *b* sequences were amplified with the primer set 258s (5′-CCATCCAACATCTCAGCATGATGAAA-3′) and 258as (5′-GCCCCTCAGAATGATATTTGTCCTCA-3′) at a 58°C annealing temperature. Amplification products had a size of 307 bp (without primer-binding sites).

Sequence analysis and phylogenetic tree construction. PCR product purification, direct sequencing with dye terminator chemistry, nucleotide sequence analysis, and amino acid sequence predictions were performed as described previously (11). Multiple-sequence alignments and phylogenetic tree construction with neighbor-joining and maximum-likelihood analysis were performed as described by Ehlers and Lowden (9).

Designation as novel rodent herpesviruses and preliminary names. All viruses were named trinomially. The first two words are the name of the host species, and the third word indicates the grouping of the novel virus within the *Herpesviridae*. All MCMV- and RCMV-like betaherpesviruses were preliminarily designated cytomegaloviruses. Although they will be most likely classified as members of the genus *Muromegalovirus*, we named them cytomegaloviruses in analogy to the name of the type species of the genus *Muromegalovirus*, *Murine cytomegalovirus* (MuHV-1). The numbering was according to the chronological order of discovery (e.g., *Apodemus flavicollis* cytomegalovirus 1).

Abbreviations use the first letter of the generic host name and the first three letters of the specific host name, followed by the abbreviation of the viral genus (e.g., AflaCMV1 [*Apodemus flavicollis* cytomegalovirus 1]).

All rodent gammaherpesviruses were preliminarily designated rhadinoviruses (e.g., *Mus musculus* rhadinovirus 1 [MmusRHV1]). The novel gammaherpesviruses described here may require the definition of additional genera within the *Gammaherpesvirinae*. This would result in changes of the provisional virus names.

Within a rodent species, DPOL sequences of less than 95% nucleic acid sequence identity were considered to be derived from different herpesvirus species, as indicated by consecutive numbering of the same virus name (e.g., BindRHV1, BindRHV2, BindRHV3, BindRHV4). Sequences of more than 95% identity were assigned to the same virus species and named identically.

Abbreviations and nucleotide sequence accession numbers. Abbreviations and accession numbers for the sequences of published viruses are as follows. (i) *Betaherpesvirinae*, genus *Cytomegalovirus*: CeHV-8 (*Cercopithecine herpesvirus 8*) = rhesus monkey cytomegalovirus (complete genome [cg], accession number [acc.] NC 006150); HHV-5 (*Human herpesvirus 5*) = HCMV (human cytomegalovirus) (cg, acc. NC 001347). (ii) *Betaherpesvirinae*, genus *Muromegalovirus*: MuHV-1 (*Murid herpesvirus 1*) = MCMV (murine cytomegalovirus) (cg, acc. NC004065); MuHV-2 (*Murid herpesvirus 2*) = RCMV-M (rat cytomegalovirus strain Maastricht) (cg, acc. NC 002512). (iii) *Betaherpesvirinae*, genus *Roseolovirus*: HHV-6 (*Human herpesvirus 6*) = HHV-6A (cg, acc. NC 001664); HHV-7 (*Human herpesvirus 7*) (cg, acc. NC 001716). (iv) *Betaherpesvirinae*, proposed genus *Proboscivirus*: ElHV-1 (*Elephantid herpesvirus 1*) = EEHV (endotheliotropic elephant herpesvirus) (partial genome, acc. AF322977). (v) *Betaherpesvirinae*, unassigned: CaHV-2 (*Caviid herpesvirus 2*) = guinea pig cytomegalovirus (complete gB and DPOL genes, acc. L25706); TuHV-1 (*Tupaiid herpesvirus 1*) = tree shrew herpesvirus (cg, acc. NC 002794); SuHV-2 (*Suid herpesvirus 2*) = PCMV (porcine cytomegalovirus) (complete gB and DPOL genes, acc. AF268039). (vi) *Gammaherpesvirinae*, genus *Lymphocryptovirus*: CalHV-3 (*Callitrichine herpesvirus 3*) (cg, acc. NC 004367); HHV-4 (*Human herpesvirus 4*) = EBV (Epstein-Barr virus) (cg, acc. NC 007605). (vii) *Gammaherpesvirinae*, genus *Rhadinovirus*: AtHV-3 (*Ateline herpesvirus 3*) = HVA (herpesvirus ateles) (cg, acc. AF083424); BoHV-4 (*Bovine herpesvirus 4*) (cg, acc. NC 002665); HHV-8 (*Human herpesvirus 8*) = KSHV (Kaposi's sarcoma-associated herpesvirus) (cg, acc. NC 003409); MuHV-4 (*Murid herpesvirus 4*) = MHV-68 (murine gammaherpesvirus 68) (cg, acc. U97553); CeHV-17 (*Cercopithecine herpesvirus 17*) = RRV (rhesus monkey rhadinovirus) (cg, acc. NC 003401); SaHV-2 (*Saimiriine Herpesvirus 2*) = HVS (herpesvirus saimiri) (cg, acc. NC 001350). (viii) *Gammaherpesvirinae*, proposed genus *Macavirus*: AlHV-1 (*Alcelaphine herpesvirus 1*) (cg, acc. NC 002531); SuHV-3 (*Suid herpesvirus 3*) = PLHV-1 (porcine lymphotropic herpesvirus 1) (partial genome, acc. AF478169). (ix) *Gammaherpesvirinae*, proposed genus *Percavirus*: EHV-2 (*Equine herpesvirus 2*) (cg, acc. NC 001650). (x) Unassigned herpesviruses: MuHV-3 (*Murid herpesvirus 3*) = MTV (mouse thymic virus); MuHV-5 (*Murid herpesvirus 5*) = *Microtus pennsylvanicus* herpesvirus; MuHV-6 (*Murid herpesvirus 6*) = sand rat inclusion agent (for MuHV-3, MuHV-5, and MuHV-6, no sequences are available in public databases).

Abbreviations and accession numbers for sequences of the novel viruses described here are listed in Tables 3 and 4.

RESULTS

General findings. Individuals of *M. musculus*, *R. norvegicus*, and 14 other rodent species were captured in several locations in Germany, the United Kingdom, and Thailand. Correct species identification was confirmed for several individuals of each rodent species by cytochrome *b* PCR (data not shown). Blood and tissue samples (n = 1,132 [including 289 brain and ganglion samples]) were first analyzed with panherpesvirus PCR targeting the DPOL gene. When no amplimer or an insufficient amount of amplimer accumulated, the PCR was repeated under more-relaxed conditions, as described in Materials and Methods. Of the 1,132 samples, 301 (27%) gave rise to an amplimer of herpesvirus origin, as revealed by sequence analysis. In 219/1,132 samples (19%) and 82/1,132 samples (7%), gammaherpesvirus and betaherpesvirus sequences, respectively, were found. No alphaherpesvirus was detected. For the purposes of this report, all novel herpesviruses were tentatively named, as described in Materials and Methods, and listed with GenBank accession numbers in Tables 3 and 4.

Genetic characterizations of novel betaherpesviruses. The betaherpesvirus DPOL sequences, found in 82 samples, originated from 19 different DPOL genes (Fig. 1C). Two of them were identical to known rodent betaherpesvirus DPOL sequences from MCMV and RCMV-E (RCMV-M was not detected). They were found in *M. musculus* and *R. norvegicus*, respectively, in accordance with published data (5, 6, 16).

The remaining 17 partial DPOL genes revealed 46% to 76% identity to MCMV DPOL (44% to 87% identity to RCMV-E and RCMV-M DPOL, respectively; values not listed) in pairwise sequence comparisons and had G+C contents of 43% to 75% (MCMV, 65%; RCMV-E, 48%; RCMV-M, 71%) (Table 3). They apparently originated from 17 novel rodent betaherpesviruses.

Next, we extended the short partial DPOL sequences toward the 5′ end of the DPOL gene and beyond, into the 3′ end of the gB gene, to allow for a more robust phylogenetic analysis. In a first step, we targeted the gB gene with degenerate primers (Fig. 1A). Using different nested-primer sets (Table 2), we amplified seven novel gB sequences (Fig. 1C). Second, we aimed to connect the gB/DPOL sequence pairs by LD-PCR (Fig. 1B), in order to confirm that the gB and DPOL sequences found in any sample originated de facto from the same virus genome. For six of the novel viruses, this approach was successful (Fig. 1C). However, it failed for AterCMV1, either because the genome copy number was insufficient to allow for successful LD-PCR or because the gB and DPOL sequences from that single sample were derived indeed from different species of betaherpesvi-

TABLE 3. Novel rodent betaherpesviruses

Origin[a]	Host		Country[b]	Virus	Abbreviation	Accession no.	% G+C content (CpG depression)	% Nucleotide sequence identity		
	Common name	Scientific name						MCMV DPOL	MCMV gB	MCMV gB + DPOL
12/9/4	Yellow-necked mouse	*Apodemus flavicollis*	GER	*Apodemus flavicollis* cytomegalovirus 1	AflaCMV1	EF125062	65 (no)	71		
2/2/2	Yellow-necked mouse	*Apodemus flavicollis*	GER	*Apodemus flavicollis* cytomegalovirus 2	AflaCMV2	EF125063	70 (no)	70	68	64
7/6/5[c]	Yellow-necked mouse	*Apodemus flavicollis*	GER	*Apodemus flavicollis* cytomegalovirus 3	AflaCMV3	EF125064	43 (no)	46	52	48
9/7/3	Bank vole	*Myodes glareolus*	GER	*Myodes glareolus* cytomegalovirus 1	MglaCMV1	EF125061	70 (no)	63	66	58
3/2/1	Common vole	*Microtus arvalis*	GER	*Microtus arvalis* cytomegalovirus 1	MarvCMV1	EF125059	72 (no)	61	73	53
1/1/1	European water vole	*Arvicola terrestris*	GER	*Arvicola terrestris* cytomegalovirus 1	AterCMV1	EF125060	75 (no)	62	74	
5/5/1	Muskrat	*Ondatra zibethicus*	GER	*Ondatra zibethicus* cytomegalovirus 1	OzibCMV1	EF125069	64 (no)	62		
1/1/1	Bandicoot rat	*Bandicota indica*	THA	*Bandicota indica* cytomegalovirus 1	BindCMV1	EF125065	51 (no)	61		
2/2/1	Bandicoot rat	*Bandicota indica*	THA	*Bandicota indica* cytomegalovirus 2	BindCMV2	EF125066	53 (no)	65		
4/3/1	Bandicoot rat	*Bandicota indica*	THA	*Bandicota indica* cytomegalovirus 3	BindCMV3	EF125067	54 (no)	66	73	65
1/1/1	Bandicoot rat	*Bandicota indica*	THA	*Bandicota indica* cytomegalovirus 4	BindCMV4	EF125068	70 (no)	71		
1/1/1	Black rat	*Rattus rattus*	THA	*Rattus rattus* cytomegalovirus 1	RratCMV1	EF125070	45 (no)	63		
2/2/1	Polynesian rat	*Rattus exulans*	THA	*Rattus exulans* cytomegalovirus 1	RexuCMV1	EF125071	57 (no)	66	77	66
2/2/1	Malaysian field rat	*Rattus tiomanicus*	THA	*Rattus tiomanicus* cytomegalovirus 1	RtioCMV1	EF125072	49 (no)	64		
1/1/1	Field vole	*Microtus agrestis*	GER	*Microtus agrestis* cytomegalovirus 1	MagrCMV1	EF125074	55 (no)	56		
2/2/1	Wood mouse	*Apodemus sylvaticus*	GER	*Apodemus sylvaticus* cytomegalovirus 1	AsylCMV1	EF125075	60 (no)	67		
1/1/1	Fawn-colored mouse	*Mus cervicolor*	THA	*Mus cervicolor* cytomegalovirus 1	McerCMV1	EF125073	70 (no)	76		
22/16/4	House mouse	*Mus musculus*	GER	Murine cytomegalovirus	MCMV	NC 004065	65 (no)	100	100	100
9/4/1	Norway rat; brown rat	*Rattus norvegicus*	GER	Rat cytomegalovirus (strain England)	RVMV-E	AY728086	48 (no)	64	76	61
	Norway rat; brown rat	*Rattus norvegicus*		Rat cytomegalovirus (strain Maastricht)	RCMV-M	NC 002512	71 (no)	71	77	63

[a] Number of samples positive by panherpesvirus DPOL PCR/number of positive individuals/number of locations.
[b] GER, Germany; THA, Thailand.
[c] The positive PCR results came from both panherpesvirus DPOL PCR and specific PCR targeting the AflaCMV3 DPOL gene.

ruses. We sequenced all LD-PCR products by primer walking, resulting in contiguous sequences of approximately 3.5 kbp for each novel virus. These encoded roughly 350 amino acids (aa) of gB and 750 aa of DPOL.

Phylogenetic analysis was performed with multiple alignments of (i) the short partial DPOL sequences (<200 bp), to include all novel viruses ($n = 17$), or (ii) concatenated gB-DPOL amino acid sequences (~1,100 aa [with gaps removed]) from all viruses for which LD-PCR was successful ($n = 6$). The first tree comprised all novel rodent viruses, the known rodent viruses MCMV, RCMV-M, and RCMV-E, and other known betaherpesviruses. Nearly all rodent viruses were found in one separate clade, consisting of four subclades: subclade I, MCMV, McerCMV1, and AflaCMV2; subclade II, RCMV-M, AflaCMV1, AsylCMV1, and BindCMV-4; subclade III, RCMV-E, RratCMV1, RratCMV2, RtioCMV1, RexuCMV1, and BindCMV1 to BindCMV3; subclade IV, AterCMV1, MglaCMV1, MagrCMV1, MarvCMV1, and OzibCMV1 (not shown). In the second tree, the same topology and higher statistical significance of branching were found (Fig. 2).

Remarkably, one virus (AflaCMV3) branched completely separately from all other rodent betaherpesviruses in both trees. In pairwise nucleic acid and amino acid sequence comparisons, it was most closely related to another rodent herpesvirus, the guinea pig betaherpesvirus (CaHV-2). In the phylogenetic tree, a clade with a multifurcation was obtained, comprising AflaCMV3, CaHV-2, TuHV-1, and three subclades (HCMV, MCMV, and HHV-6A, with their relatives, respectively). Therefore, its evolutionary relatedness remained somewhat uncertain (Fig. 2). To confirm that *A. flavicollis* is the natural host for AflaCMV3, 31 individuals were analyzed with primers (3838-s/3838-as) specific for the AflaCMV3 DPOL gene. Six spleen, lung, and kidney samples (about 20%) were positive (Table 3).

Genetic characterizations of novel gammaherpesviruses. The gammaherpesvirus DPOL sequences, detected in 219 samples with degenerate primers, originated from 22 different DPOL genes. One of them was identical to the known MHV-68 DPOL gene and was found in three *Apodemus* species, namely, *A. sylvaticus*, *A. flavicollis*, and *A. agrarius* (but not in *M. glareolus*, from which MHV-68 was originally isolated [4]). The other partial DPOL genes appeared to originate from 21 as-yet-unknown rodent gammaherpesviruses. Upon analysis

TABLE 4. Novel rodent gammaherpesviruses

Origin[a]	Host		Country[b]	Virus	Abbreviation	Accession no.	% G+C content (CpG depression)	% Nucleotide sequence identity		
	Common name	Scientific name						MHV-68 DPOL	MHV-68 gB	MHV-68 gB + DPOL
75/33/3[c]	House mouse	*Mus musculus*	GER/UK	*Mus musculus* rhadinovirus 1	MmusRHV1	AY854167	65 (no)	45	58	53
12/5/1	Fawn-colored mouse	*Mus cervicolor*	THA	*Mus cervicolor* rhadinovirus 1	McerRHV1	DQ821582	64 (no)	45	60	54
8/4/1	Norway rat; brown rat	*Rattus norvegicus*	GER	*Rattus norvegicus* rhadinovirus 1	RnorRHV1	EF128038	69 (no)	41	54	
1/1/1	Norway rat; brown rat	*Rattus norvegicus*	GER	*Rattus norvegicus* rhadinovirus 2	RnorRHV2	EF128039	69 (no)	45		
2/2/1	Bandicoot rat	*Bandicota indica*	THA	*Bandicota indica* rhadinovirus 1	BindRHV1	EF128040	66 (no)	41		
3/3/1	Bandicoot rat	*Bandicota indica*	THA	*Bandicota indica* rhadinovirus 2	BindRHV2	EF128041	70 (no)	44	57	
1/1/1	Bandicoot rat	*Bandicota indica*	THA	*Bandicota indica* rhadinovirus 3	BindRHV3	EF128042	70 (no)	43		
1/1/1	Bandicoot rat	*Bandicota indica*	THA	*Bandicota indica* rhadinovirus 4	BindRHV4	EF128043	40 (yes)	58	70	67
6/3/1	Lesser bandicoot rat	*Bandicota savilei*	THA	*Bandicota savilei* rhadinovirus 1	BsavRHV1	DQ821581	64 (no)	41	56	50
15/9/1	Black rat	*Rattus rattus*	THA	*Rattus rattus* rhadinovirus 1	RratRHV1	EF128044	70 (no)	41		
5/3/1	Black rat	*Rattus rattus*	THA	*Rattus rattus* rhadinovirus 2	RratRHV2	EF128045	70 (no)	43		
3/3/1	Black rat	*Rattus rattus*	THA	*Rattus rattus* rhadinovirus 3	RratRHV3	EF128046	66 (no)	40	53	
2/1/1	Polynesian rat	*Rattus exulans*	THA	*Rattus exulans* rhadinovirus 1	RexuRHV1	EF128047	66 (no)	40		
1/1/1	Polynesian rat	*Rattus exulans*	THA	*Rattus exulans* rhadinovirus 2	RexuRHV2	EF128048	69 (no)	40	59	
2/2/1	Malaysian field rat	*Rattus tiomanicus*	THA	*Rattus tiomanicus* rhadinovirus 1	RtioRHV1	EF128049	45 (yes)	60		
1/1/1	Malaysian field rat	*Rattus tiomanicus*	THA	*Rattus tiomanicus* rhadinovirus 2	RtioRHV2	EF128050	69 (no)	42		
16/5/2	Striped field mouse	*Apodemus agrarius*	GER	*Apodemus agrarius* rhadinovirus 1	AagrRHV1	AY854170	58 (no)	50		
9/7/2	Yellow-necked mouse	*Apodemus flavicollis*	GER	*Apodemus flavicollis* rhadinovirus 1	AflaRHV1	DQ821580	47 (yes)	85	90	89
13/17/2	Wood mouse	*Apodemus sylvaticus*	GER/UK	*Apodemus sylvaticus* rhadinovirus 1	AsylRHV1	EF128051	47 (yes)	85	91	90
9/7/3	Bank vole	*Myodes glareolus*	GER	*Myodes glareolus* rhadinovirus 1	MglaRHV1	AY854169	40 (yes)	56	72	61
12/7/2	Field vole	*Microtus agrestis*	GER/UK	*Microtus agrestis* rhadinovirus 1	MagrRHV1	EF128052	49 (yes)	58	70	62
27/11/3	Wood mouse; striped field mouse; yellow-necked mouse	*Apodemus sylvaticus*; *A. flavicollis*; *A. agrarius*	GER	Murine gammaherpesvirus 68	MHV-68	NC_001826	50 (yes)	100	100	100

[a] Number of samples positive by panherpesvirus DPOL PCR/number of positive individuals/number of locations.
[b] GER, Germany; UK, United Kingdom; THA, Thailand.
[c] The positive PCR results came from both panherpesvirus DPOL PCR and specific PCR targeting the MmusRHV1 gB gene.

of their G+C contents and CpG dinucleotide suppression (12), they fell into two groups. Group I had a G+C content of 40% to 49% (MHV-68, 50%) and a clearly visible CpG suppression (like MHV-68). Group II had a G+C content of 58% to 70% and no CpG suppression (Table 4). Group I comprised MHV-68 and novel viruses of *Apodemus flavicollis* (AflaRHV1), *Apodemus sylvaticus* (AsylRHV1), *Myodes glareolus* (MglaRHV1), *Microtus agrestis* (MagrRHV1), *Bandicota indica* (BindRHV4), and *Rattus tiomanicus* (RtioRHV1). Group II comprised a novel rhadinovirus of *Mus musculus* (MmusRHV1) and those of *Mus cervicolor* (McerRHV1), *Apodemus agrarius* (AagrRHV1), *Rattus norvegicus* (RnorRHV1-2), *Rattus rattus* (RratRHV1-3), *Rattus exulans* (RexuRHV1-2), *Rattus tiomanicus* (RtioRHV2), *Bandicota indica* (BindRHV1-3), and *Bandicota savilei* (BsavRHV1). Interestingly, representatives of both groups were detected in the same rodent species, i.e., *B. indica*, *R. tiomanicus*, and *A. agrarius*.

Pairwise comparisons of partial DPOL sequences revealed 58% to 85% identity (group I) and 40% to 50% identity (group II) to the corresponding MHV-68 sequence. Both the group I and group II viruses revealed a slightly higher percentage of identity to HHV-8 than to EBV. Therefore, all were tentatively designated rhadinoviruses.

Phylogenetic analysis was performed with multiple alignments of (i) the short partial DPOL sequences of all novel gammaherpesviruses ($n = 21$) or (ii) concatenated gB-DPOL amino acid sequences ($n = 8$), with gaps removed. In both trees, two distantly related clades resulted. Clade I comprised MHV-68 and the other viruses of group I. Clade II included the novel virus of the house mouse (MmusRHV1) and the other group II viruses (Fig. 3).

MmusRHV1 and all other group II viruses clearly differed from MHV-68, because they revealed percentages of identity between 45% and 55% in comparison to MHV-68 on the nucleotide and amino acid levels. Beyond this, the phylogenetic place-

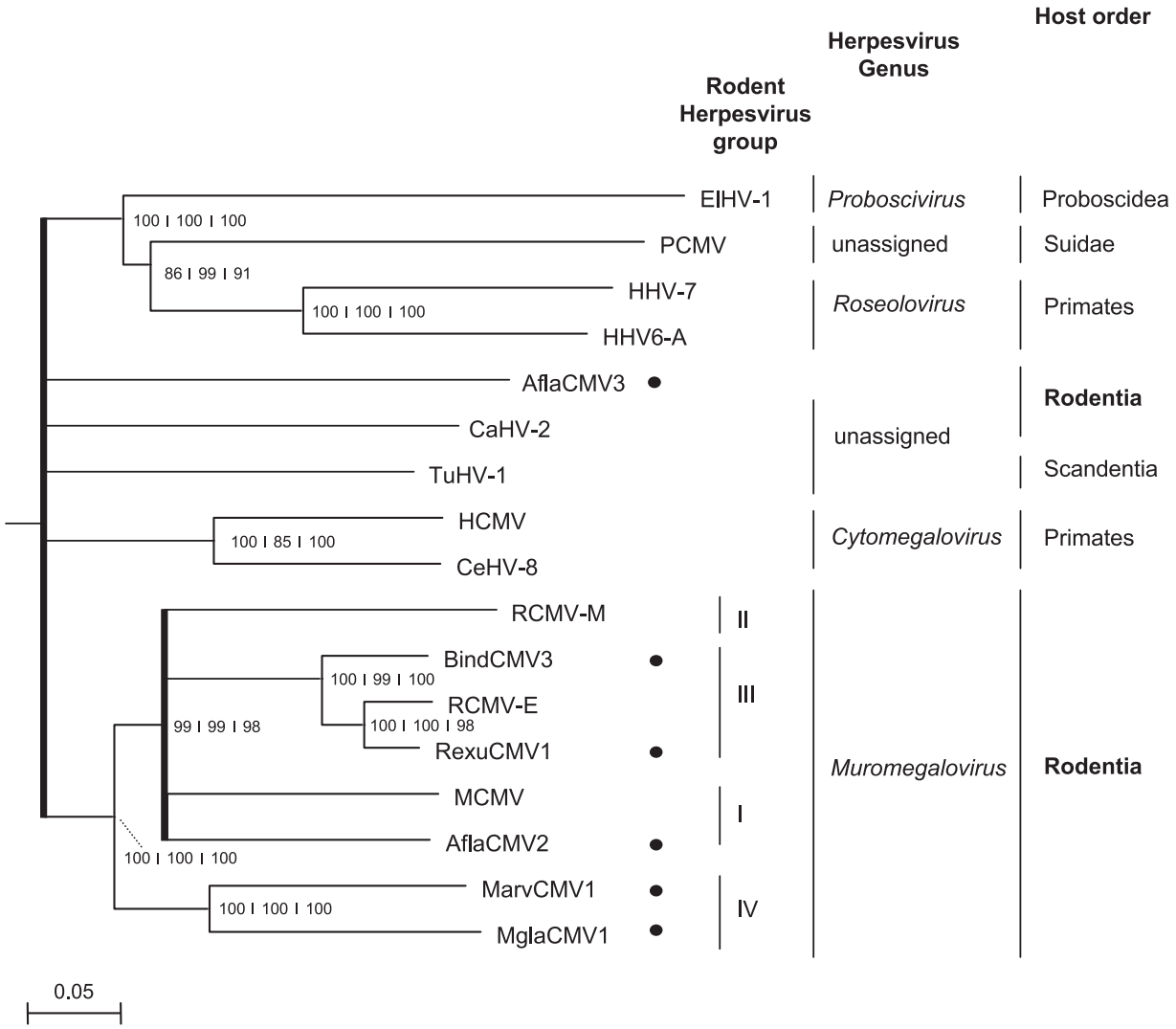

FIG. 2. Phylogenetic analysis of novel rodent betaherpesviruses. A phylogenetic tree was constructed by using the amino acid sequences encoded by the gB-DPOL segments of the novel rodent betaherpesviruses (Table 3) and of known betaherpesviruses, available in the GenBank database. A multiple alignment of 1,100 aa (concatenated) was analyzed by the neighbor-joining method of the program MacVector (version 8.0). A midpoint-rooted phylogram is shown. The branch length is proportional to evolutionary distance (scale bar). Results of bootstrap analysis (100-fold) are indicated at the nodes of the tree to the left of the first vertical divider. In addition, the alignment was analyzed with the program TREE-PUZZLE (version 5.0). The tree topology was the same (not shown). Support values, estimated by the quartet puzzling (QP) tree search and expressing QP reliability (in percentages), are indicated to the left of the second vertical divider. In addition, the alignment was analyzed by the maximum-likelihood method of the PHYLIP program package (version 3.65). The results of bootstrap analysis (100-fold) are presented to the right of the second vertical divider. Nodes that did not appear consistently in all analyses or that had bootstrap values mainly below 70% are masked with a thick black bar. The novel viruses are marked with black oval symbols. Herpesvirus genera and groups I to IV of the members of the genus *Muromegalovirus*, as well as the host orders, are indicated. Abbreviations of viruses are listed with full virus names in Materials and Methods and in Table 3.

ment of the group I and group II clades within the *Gammaherpesvirinae* was uncertain, even in the 1,100-aa tree (Fig. 3).

For a more meaningful phylogenetic analysis of MmusRHV1, we further extended the MmusRHV1 sequence from open reading frame 8 (ORF8), gB, through ORF7 and into ORF6, encoding the major DNA-binding protein. This was achieved with degenerate PCR targeting ORF6 and subsequent LD-PCR between ORF6 and ORF8. A final contiguous sequence of approximately 8 kbp was generated, extending from ORF6, MDBP, to ORF9, DPOL. A phylogenetic tree was constructed with >3,000 aa, including the EBV-like lymphocryptoviruses, the HHV-8-like rhadinoviruses, and the AlHV-1-like members of the proposed genus *Macavirus*. Again, MmusRHV1 branched distantly from MHV-68. A multifurcated clade was obtained, comprising Mmus RHV1 and three subclades (*Rhadinovirus*, *Percavirus*, and *Macavirus* species) (Fig. 4).

Finally, the occurrence of MmusRHV1 in the blood of free-living rodents was analyzed by specific PCR. For this purpose, 104 house mice were trapped in the United Kingdom, and samples were tested with nested PCR specific for the MmusRHV1 gB gene. Five samples (about 5%) were positive for MmusRHV1 (Table 4). In contrast, 26 wood mice (*A. sylvaticus*), 11 bank voles (*M. glareolus*), and 77 field voles (*M. agrestis*) from the United Kingdom were

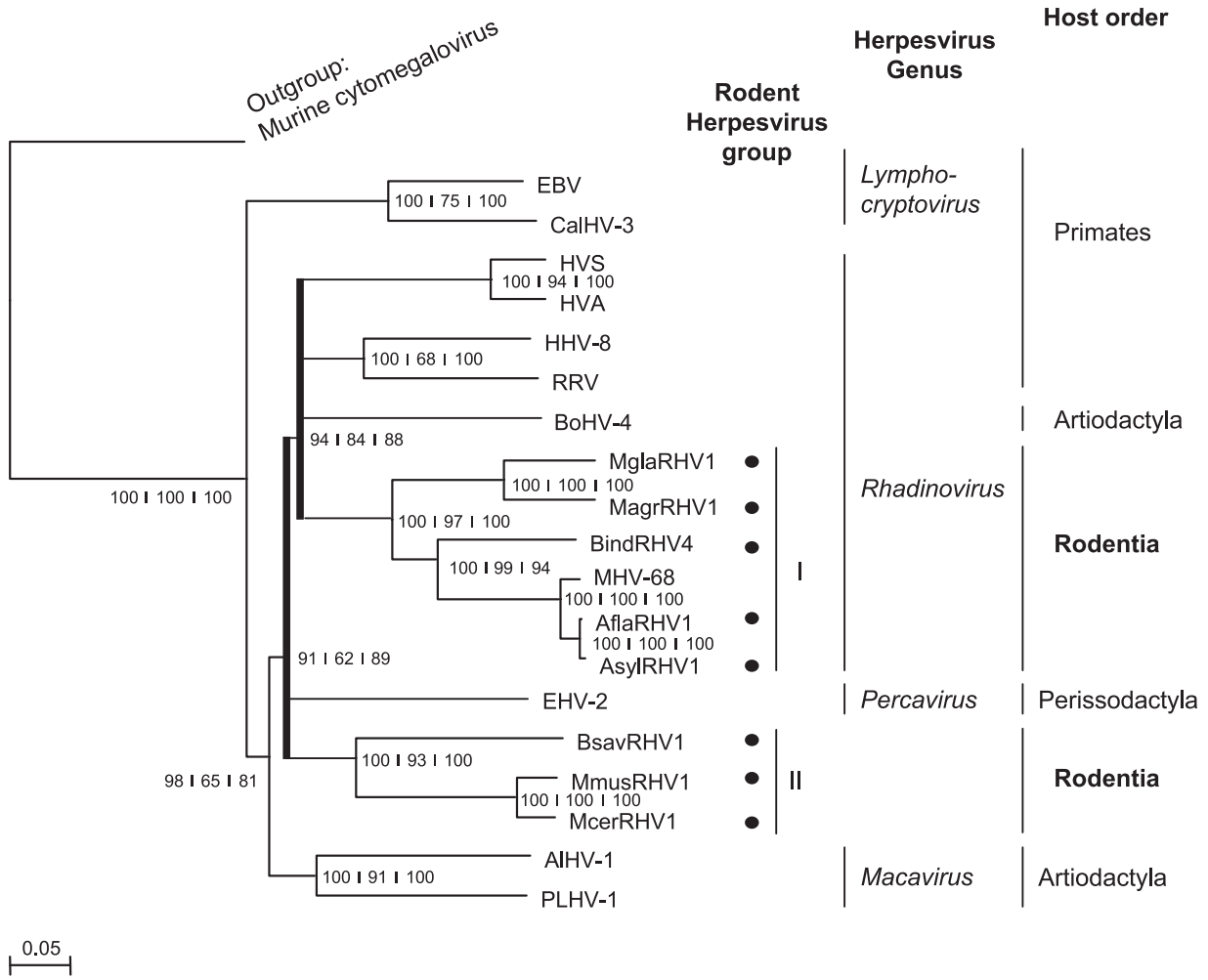

FIG. 3. Phylogenetic analysis of novel rodent gammaherpesviruses. A phylogenetic tree was constructed by using the amino acid sequences encoded by the gB-DPOL fragments of the novel rodent gammaherpesviruses (Table 4) and of known gammaherpesviruses, available in the GenBank database. MCMV was used as the outgroup. A multiple alignment of 1,100 aa (concatenated) was analyzed as described in legend to Fig. 2. The novel viruses are marked with black oval symbols. Herpesvirus genera and groups I to II of the rodent rhadinoviruses, as well as the host orders, are indicated. Abbreviations of viruses are listed with full virus names in Materials and Methods and in Table 4.

negative for MmusRHV1 in spleen samples (blood samples were not available).

DISCUSSION

Here, we present results of the first comprehensive search for alpha-, beta-, and gammaherpesviruses in *M. musculus*, *R. norvegicus*, and other rodents, using PCR approaches that target different conserved genes. The study was based on >1,000 samples, including 289 brain and ganglion samples. It revealed a plethora of novel beta- and gammaherpesviruses.

Amplification of the gB gene and subsequent LD-PCR, spanning the gB-DPOL segment, was successful for one-third ($n = 14$) of these novel viruses. The most likely reason for the missing amplification of the gB gene in the remaining viruses is that gB is not conserved enough to allow for the amplification of all vertebrate herpesvirus gB genes with a limited number of degenerate primer sets (as is possible with the DPOL gene). Therefore, binding with low specificity of one or more gB primers or a complete absence of binding may have occurred. In addition, several samples probably contained an insufficient genome copy number.

Seventeen novel rodent betaherpesviruses were identified. Sixteen of these clustered with MCMV and the RCMV strains RCMV-M and RCMV-E, forming a large, separate clade with four subclades (Fig. 2). This analysis also showed that RCMV-M and RCMV-E are separate species, in line with the remarkable differences in their individual gene contents, as reported previously (27, 2). In *Peromyscus maniculatus* (deer mouse), as well as in *R. norvegicus* and *R. rattus*, several cytomegaloviruses have been detected recently. It remains to be determined how these relate to the rodent viruses described here, because only short partial sequences of the ORFs of UL33 (*P. maniculatus*) and R87 (*Rattus* species) have been published (22, 18).

Among the novel betaherpesviruses, AflaCMV3 was unique because it did not cluster closely with the MCMV- and RCMV-

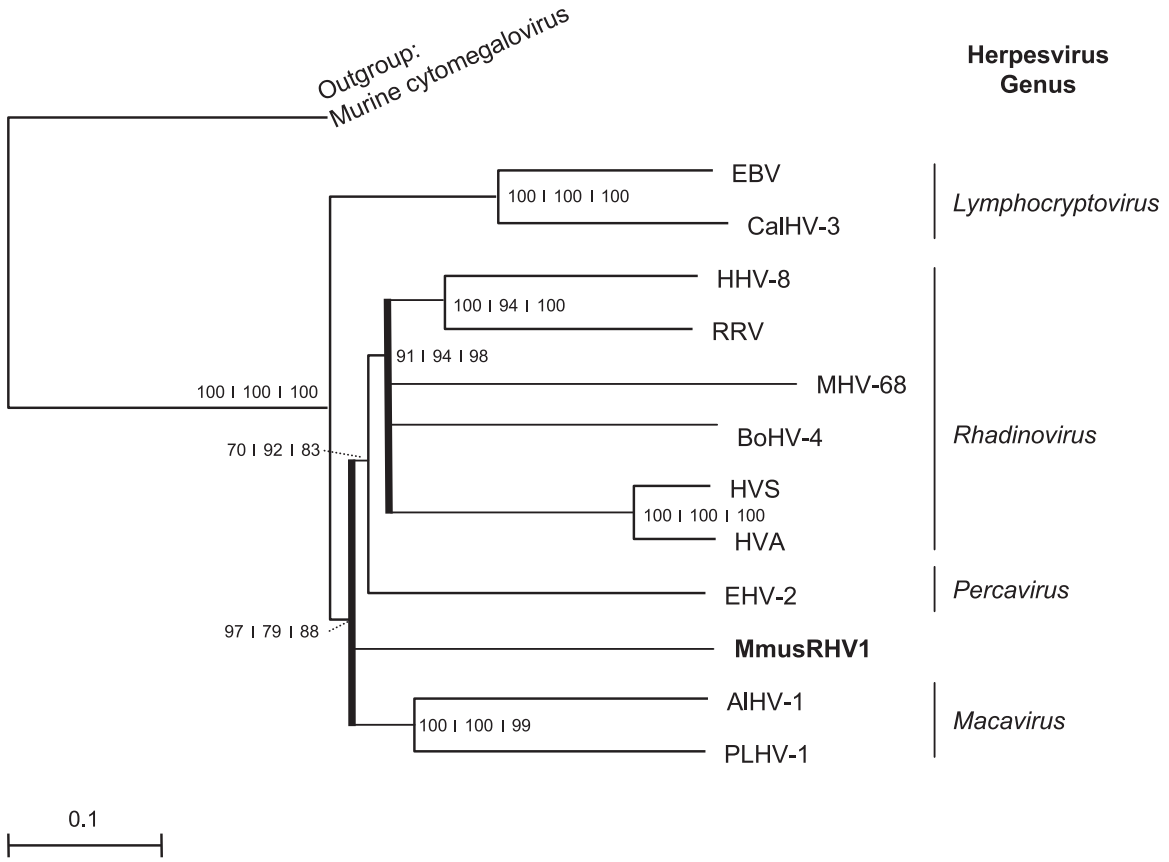

FIG. 4. Extended phylogenetic analysis of the novel virus MmusRHV1. A phylogenetic tree was constructed by using the amino acid sequences encoded by the MDBP-DPOL fragment of MmusRHV1 and of known gammaherpesviruses, available in the GenBank database. MCMV was used as the outgroup. A multiple alignment of >3,000 aa (concatenated) was analyzed as described in the legend to Fig. 2. MmusRHV1 is indicated in boldface type. Herpesvirus genera are indicated.

related cytomegaloviruses, including AflaCMV1 and AflaCMV2, which originated from the same host (*A. flavicollis*). As soon as the isolation and propagation of AflaCMV3 in tissue culture succeeds, its repertoire of nonconserved genes can be compared with those of the human betaherpesviruses and assessed for its suitability in model studies.

No evidence of alphaherpesviruses in rodents was obtained. Several reasons may account for this failure. Their prevalence may be quite low, preventing detection in a limited number of samples, and, as their loci of latency are unknown, we may have missed them through our choice of specimens. In addition, the primers used for universal detection of herpesviruses may sufficiently bind to and amplify rodent alphaherpesvirus DPOL genes only in samples with high viral loads. It is possible that such samples might be obtained only under certain rare disease conditions. However, it is also possible that rodent alphaherpesviruses either never developed or became extinct earlier during herpesvirus evolution. Further studies are needed to clarify this issue.

We identified 21 novel rodent gammaherpesviruses. They clearly clustered into two different groups (I and II), as revealed by the differences in (i) their sequences, (ii) their G+C contents, and (iii) the presence of CpG suppression (Table 4), as well as (iv) their phylogeny (Fig. 3). The most interesting member of group II is MmusRHV1. It is the first gammaherpesvirus of *M. musculus* and was detected in 75 samples (31% of all tested samples) from 33 individuals of *M. musculus* in Germany and the United Kingdom but not in organs of the other rodent species. These data firmly indicate that MmusRHV1 is the first gammaherpesvirus that naturally infects *M. musculus*. The comparatively low frequency of MmusRHV1 detection in blood samples (roughly 5%) most likely reflects the biology of the virus. Virus may reside (and be detected) in the spleens of all infected animals but may be detected in only a small proportion of blood samples, as is the case for mice experimentally infected with MHV-68 (24).

Rowe and Capps (19) discovered a mouse virus that causes thymic necrosis in newborn mice (*M. musculus*). This virus exhibits T-cell tropism, persists in salivary glands, and could not be propagated in cell culture. It was named mouse thymic virus (MTV) and classified as MuHV-3. Our recent seroepidemiological studies have shown that MuHV-3 (like MCMV) has ubiquitous presence in free-living European house mice (1). We were therefore concerned that MmusRHV1 might in fact be MuHV-3. No sequences of MuHV-3 are available in public databases, but recently a partial DPOL sequence of MuHV-3 was amplified and found to be betaherpesvirus-like (R. S. Livingston, University of Missouri—Columbia, personal

communication). Therefore, MmusRHV1 and MuHV-3 are completely different herpesvirus species.

MHV-68 infection of laboratory mice is extensively studied in the pursuit of insight into gammaherpesvirus pathogenesis. However, its natural hosts are *M. glareolus* (4) and several *Apodemus* species (3; also this study), the latter being in accordance with the close phylogenetic relationship of MHV-68 to AflaRHV1 and AsylRHV1 (Fig. 3). In addition, MHV-68 infection fails to reproduce all aspects of human gammaherpesvirus disease. Since laboratory mice are originally derived from *M. musculus*, MHV-68 has not been studied in its natural host. The investigation of MmusRHV1 (from *M. musculus*) or its close relative McerRHV1 (from *M. cervicolor*) in laboratory mice may result in data that reflect more reliably gammaherpesvirus infection in nature. In addition, it may facilitate the study of aspects of human gammaherpesvirus disease that are not visible in the MHV-68 model. MmusRHV1 and Mcer RHV1 branch distantly from MHV-68 within the *Gammaherpesvirinae*. Therefore, their content of individual (nonconserved) genes may differ considerably from that of MHV-68. Such genes represent the individual makeup of each herpesvirus and are the basis for their unique pathogenic properties. To characterize their genomes and study their biology, Mmus RHV1 (and/or McerRHV1) will have to be isolated and propagated in tissue culture.

The rodent herpesviruses described here for the first time add significant data to herpesvirus phylogeny, and their further characterization will amend our understanding of herpesvirus biology. In particular, MmusRHV1, the first gammaherpesvirus to naturally infect *M. musculus*, and the related virus in *M. cervicolor* (McerRHV1), as well as the novel betaherpesvirus AflaCMV3, will be explored for potential as experimental tools for the study of beta- and gammaherpesvirus pathogenesis.

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